AD_____

Award Number: W81XWH-11-1-0098

TITLE: Measurement of S-nitrosylated Proteins in Tissues of Rats Fed Diets with Differing Levels of Nitrite

PRINCIPAL INVESTIGATOR: Dr. Valerian Kagan

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, PA 15213

REPORT DATE: December 2011

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-12-2011	Final	1 DEC 2010 - 30 NOV 2011

4. TITLE AND SUBTITLE
Measurement of S-nitrosylated Proteins in Tissues of Rats Fed Diets with Differing Levels of Nitrite

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0098

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dr. Valerian Kagan

E-Mail: kagan@pitt.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pittsburgh
Pittsburgh, PA 15213

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Increased ventilation is a general response to shock that may help to maintain cardiac output by improving venous return to the heart. The central stimulus for this increase in ventilation involves the production of S-nitroso-L-cysteine from more ubiquitous precursor S-nitrothiols in the medulla of the brain. Changes in dietary nitrite and nitrate have been shown to increase tissue levels of S-nitrosothiols and to improve survival in models of myocardial infarction. We hypothesize that increased dietary intake of nitrite or nitrate will enhance the ventilatory response in hemorrhage and thereby increase the time of survival. The goal of this project was to adequate methodology for the detection of S-nitrosylated proteins in tissues. A sensitive and specific method for the detection of S-nitrosylated proteins in tissues based on photolysis/immuno-spin-trapping analysis of DMPO thioethers has been developed. The applicability of the method for the quantitative assessments of Snitrosylated proteins in tissues has been tested and confirmed. The ability of dihydrolipoic acid and lipoic acid (LA) plus lipoamide dehydrogenase and NADH to denitrosate S-nitrosocaspase 3 (CASP-SNO) and regulate apoptosis in HepG2 cells has been established.

15. SUBJECT TERMS
Protein nitrosylation, S-nitroso-proteins, immune-spin trapping, spin trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO)

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	9	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Introduction……..…….…... 3

BODY…….. 4

Key Research Accomplishments…….…….. 6

Reportable Outcomes…… 7

Conclusion…… 7

References…….. 7

Appendices (Literature cited) …… 8

Introduction:

According to the work accomplished by Dr. James Atkins at Walter Reed Army Institute of Research, early survival in hemorrhagic shock can be influenced by changes in dietary nitrite or nitrate. In particular, changes in dietary nitrite/nitrate influence the early ventilatory response to hemorrhage. Increased ventilation is a general response to shock that helps to maintain cardiac output by improving venous return to the heart. The ventilatory response occurs very early during hemorrhage and it is possible that preserved cardiac output during this critical time period would allow more casualties to survive until the medics could arrive to place life-saving tourniquets.

The rationale and background for the ventilation studies were presented in detail in the grant proposal, which has been funded by the Office of Naval Research (#42237). The hypothesis is that nitrite is converted to nitric oxide (NO), a small S-nitrosothiol, S-nitrosoglutathione (GSNO), and a number of S-nitrosylated proteins (RSNOs). Both NO and S-nitrosothiols are known to affect the generation and transmission of the neuronal signals controlling ventilation (Diers et al., 2011; Prime et al., 2009; Palmer, 2006). GSNO is in equilibrium with S-nitrosylated proteins (SNO proteins). Because RSNO proteins are more stable than GSNO they are more accurately measured. Moreover RSNO proteins themselves are known to serve important regulatory functions in a wide-range of cellular pathways (Lima et al., 2010; Nagahara et al., 2010).

Our *major goal was to develop a novel specific and sensitive method to measure RSNO proteins in tissues.*

BODY:

Background:

Recent studies have shown that increased dietary intake of nitrite or nitrate affords protection in myocardial ischemia reperfusion through an increase in the production of nitric oxide. It is possible that increased dietary intake of nitrate or nitrite might also improve survival in severe hemorrhage through an increase in ventilation. Increased ventilation is a general response to shock that helps to maintain cardiac output by improving venous return to the heart. Nitric oxide and it products have a large influence on the neural control of breathing and increased levels may enhance the normal ventilatory response allowing casualties to survive longer in the face of severe hemorrhage. The half-life of nitric oxide is quite short and evidence of increased production can be assessed better by measurement of more stable products such as S-nitrosylated proteins.

Hypothesis:

Immuno-spin trapping protocol can be applicable for the detection of S-nitrosylated proteins in the liver and brain of rats exposed to dietary manipulated nitrite/nitrate

Specific Aims:

1. Develop a sensitive and specific method for the detection of S-nitrosylated proteins in tissues.

2. Apply this new methodology to the evaluation of S-nitrosothiols in tissue samples provided by Dr. Atkins. Overall, there were 4 groups of rats (1-control; 2-low nitrite/nitrate diet for 7 days; 3-low N/N diet supplemented with nitrate in the drinking water for 7 days; 4- low N/N diet supplemented with nitrite in the drinking water for 7 days).

Study Design:

The accomplished work complemented a study by Dr. James Atkins at Walter Reed Army Institute of Research. Ventilation/hemorrhage studies, tissue collection and the measurement of tissue nitrite have been performed by Dr. Atkins under an animal use protocol approved at Walter Reed Army Institute of Research # M04-08, under a grant funded by the Office of Naval Research. The tissue samples have been analyzed by Dr. Kagan's lab utilizing the newly developed methodology. In brief, tissue extracts were irradiated with a bright UV light in the presence of a spin trap, 5,5-dimethyl-1-pyrroline N-oxide, which under these conditions binds to the S-nitrosylated proteins with a covalent bond. This tag has been used to quantitate the S-nitrosylated proteins by Western blotting using anti-DMPO antibody.

Relevance:

The measurement of S-nitrosylated proteins can provide an important evidence that the dietary manipulations have resulted in changes in nitric oxide production and metabolism. The importance of the overall project could be substantial. Excluding immediate deaths, 50% of all battlefield deaths (KIAs) occur within 5 minutes of injury, prior to the arrival of the medic. An enhanced ventilatory response may allow more casualties to survive until the medic can arrive to place life-saving tourniquets.

Key Research Accomplishments:

1. Currently, the spin trapping of S-centered radicals with DMPO is the paramount method for analysis of these radical species. DMPO has a well established mechanistic and derivative chemistry, and an array of optimized analytical methods exists for quantitation of DMPO thioethers. Furthermore, DMPO exhibits low cytotoxicity. In spin trapping experiments, cells tolerate this nitrone in concentrations up to 0.1 M, which makes it suitable for analysis of RS• in intact cells. Hence, the protocols for assessment of DMPO thioethers represent a unique approach that allows reliable analysis of the biochemistry and pathology of radical-mediated protein oxidation and/or of S-nitrosation. Based on these innovative ideas, a sensitive and specific methodology has been developed for the detection of changes in the content of S-nitrosylated proteins (PSNOs). The protocol is based on photolysis/immuno-spin-trapping analysis of DMPO thioethers. Our experiments proved its applicability for the purpose of analysis of S- nitrosylated proteins in biochemical systems as well as in tissues (Stoyanovsky et al., 2011).

2. Nitric oxide (NO), produced either extracellularly by low molecular mass (LMM) compounds or intracellularly by nitric oxide synthases, impedes activity of caspases – critical participants of cell death pathways - via reactions of S-nitrosation. Caspases can undergo poly-S-nitrosation, whereby all SNO functions in its p12 subunit are denitrosated by reduced glutathione (GSH) except for a single SNO group (Diers et al., 2011; Prime et al., 2009; Palmer, 2006). Since the latter was not observed in a mutant form of CASP-SH lacking the active site cysteine, Zech et al. proposed that in cells NO nitrosates this cysteine to form S-nitrosocaspase 3 (CASP-SNO) that is resistant to reduction by GSH (Diers et al., 2011; Prime et al., 2009; Palmer, 2006). However, denitrosation of CASP-SNO back to CASP-SH with reconstitution of its proteolytic activity is catalyzed in cellular cytosol. We found that that dihydrolipoic acid and lipoic acid (LA) plus lipoamide dehydrogenase and NADH denitrosate S-nitrosocaspase 3 (CASP-SNO). In HepG2 cells, S-nitroso-L-cysteine ethyl ester (SNCEE) impeded the activity of caspase 3 (CASP-SH), while a subsequent incubation of the cells in SNCEE-free medium resulted in endogenous denitrosation and reactivation of CASP- SH. The latter process was

inhibited in thioredoxin reductase-deficient HepG2 cells, in which, however, LA markedly reactivated CASP-SH. The data obtained are discussed with focus on low molecular mass dithiols that mimic the activity of thioredoxin in reactions of protein S-denitrosation.

Reportable outcomes:

- A sensitive and specific method for the detection of S-nitrosylated proteins in tissues based on photolysis/immuno-spin-trapping analysis of DMPO thioethers has been developed.
- The applicability of the method for the quantitative assessments of S-nitrosylated proteins in tissues has been tested and confirmed.
- The ability of dihydrolipoic acid and lipoic acid (LA) plus lipoamide dehydrogenase and NADH to denitrosate S-nitrosocaspase 3 (CASP-SNO) and regulate apoptosis in HepG2 cells has been established.

Conclusion: A novel sensitive and specific method for the detection of S-nitrosylated proteins based on photolysis/immuno-spin-trapping analysis of DMPO thioethers can be applied for the assessments of these important metabolites in tissues.

References – The results of the work performed have been published in two peer-reviewed papers and presented at one National Meeting as indicated below:

Sengupta R, Billiar TR, Atkins JL, Kagan VE, Stoyanovsky DA. Nitric oxide and dihydrolipoic acid modulate the activity of caspase 3 in HepG2 cells. FEBS Lett. 2009;583(21):3525-30.

Stoyanovsky DA, Maeda A, Atkins JL, Kagan VE. Assessments of thiyl radicals in biosystems: difficulties and new applications. Anal Chem. 2011;83(17):6432-8

Atkins JL, Kagan V, Byzek S, Keesee J, Gist I and Lu X Enhanced survival in hemorrhagic shock through changes in dietary nitrite. (2009) Advanced Technology Applications for Combat Casualty Care (ATACCC) Conference, TradeWinds Island Grand Resort, August 10 - 12, 2009, St. Pete Beach, Florida.

Literature cited:

Diers AR, Broniowska KA, Darley-Usmar VM, Hogg N. Differential regulation of metabolism by nitric oxide and S-nitrosothiols in endothelial cells. Am J Physiol Heart Circ Physiol. 2011;301(3):H803-12.

Lima B, Forrester MT, Hess DT, Stamler JS. S-nitrosylation in cardiovascular signaling. Circ Res. 2010; 106(4):633-46.

Nagahara N, Matsumura T, Okamoto R, Kajihara Y. Protein cysteine modifications: (1) medical chemistry for proteomics. Curr Med Chem. 2009;16(33):4419-44

Prime TA, Blaikie FH, Evans C, Nadtochiy SM, James AM, Dahm CC, Vitturi DA, Patel RP, Hiley CR, Abakumova I, Requejo R, Chouchani ET, Hurd TR, Garvey JF, Taylor CT, Brookes PS, Smith RA, Murphy MP. A mitochondria-targeted S-nitrosothiol modulates respiration, nitrosates thiols, and protects against ischemia-reperfusion injury. Proc Natl Acad Sci U S A. 2009;106(26):10764-9.

Palmer LA. Regulation of respiration and endothelial gene expression by S-nitrosothiols in health and disease. Proc Am Thorac Soc. 2006;3(2):166-9

Stoyanovsky DA, Maeda A, Atkins JL, Kagan VE. Assessments of thiyl radicals in biosystems: difficulties and new applications. Anal Chem. 2011;83(17):6432-8